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Repeated Inhalation Exposure of FE-13 in Mice, Mus musculus (Bone Marrow Micronucleus Assay)

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Integrated Laboratory System
800-12, Capital drive
Drum, NC 27713

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U.S Army Center for Health Promotion & Preventive Medicine
Aberdeen Proving Ground, MD 21010

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14. ABSTRACT
Repeated inhalation with the test article FE-13 (Tifluoromethane) did not significantly increase the frequency of micronucleated PCEs in the bone marrow of male or female B6C3F1 mice and or significantly affect the percentage of PCEs in either sex.

15. SUBJECT TERMS
FE-13, Trifluoromethane, Mice, Micronucleus Assay, Bone Marrow.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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Integrated Laboratory Systems

STUDY TITLE

Repeated Inhalation Exposure of FE-13 in Mice, *Mus musculus*
(Bone Marrow Micronucleus Assay)

Project No.
ILS A073-002

Sponsor's Contract Number
DAAD05-91C-0018

U.S. Army Study Number
85-3587

Test Article
FE-13

Final Report Date
January 19, 1996

Sponsor
U. S. Army Center for Health Promotion and Preventative Medicine
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QUALITY ASSURANCE INSPECTION STATEMENT

ILS Project No.: A073-002
Test Article ID: FE-13
ILS Repository No.: Not Applicable
Study Title: Repeated Inhalation Exposure of FE-13 in Mice, *Mus musculus* (Bone Marrow Micronucleus Assay)

This study was inspected by the Quality Assurance Unit of Integrated Laboratory Systems, Research Triangle Park, NC, and written status reports were submitted on the following dates:

Inspection/Audit	Date Performed	Date Reported to Study Director/Management	
Study Protocol	8/18/95	8/18/95	8/22/95
Slide Scoring	1/10/96	1/10/96	1/17/96
Data Audit	1/18/96	1/19/96	1/19/96
Final Report Audit	1/18/96	1/19/96	1/19/96

Kaye Cummings
Kaye Cummings, B.S.
Quality Assurance Officer

1/19/96
Date

CERTIFICATION OF GOOD LABORATORY PRACTICE

ILS Project No.:	A073-002
Test Article ID:	FE-13
ILS Repository No.:	Not Applicable
Study Title:	Repeated Inhalation Exposure of FE-13 in Mice, *Mus musculus* (Bone Marrow Micronucleus Assay)

This study was conducted in accordance with Good Laboratory Practice regulations as promulgated by the U.S. Environmental Protection Agency (40 CFR Part 792).

Raymond Tice
Raymond R. Tice, Ph.D.
Study Director

1-19-96
Date

CERTIFICATION OF CONTRACT COMPLIANCE

ILS Project No.: A073-002
Test Article ID: FE-13
ILS Repository No.: Not Applicable
Study Title: Repeated Inhalation Exposure of FE-13 in Mice, *Mus musculus* (Bone Marrow Micronucleus Assay)

The contractor, Integrated Laboratory Systems, hereby certifies that, to the best of its knowledge and belief, the technical data delivered herewith under Contract No. DAAD05-91-C-0018 is complete, accurate, and complies with all requirements of the contract.

Raymond Tice
Raymond R. Tice, Ph.D.
Study Director

1-19-96
Date

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FINAL REPORT

1.0 Study Title:

Repeated Inhalation Exposure of FE-13 in Mice, *Mus musculus*
(Bone Marrow Micronucleus Assay)

2.0 Study Identification:

ILS Project No. A073-002
Sponsor's Contract No. DAAD05-91-C-0018
U.S. Army Study No. 85-3587

3.0 Purpose of the Study:

The objective of this study is to evaluate the polychromatic erythrocytes (PCE) of male and female B6C3F1 mice exposed via repeated inhalation to FE-13 (trifluoromethane) for the presence of micronuclei.

4.0 Test Article:

FE-13

5.0 Names of Study Director and Sponsor Study Monitor:

Study Director: Raymond R. Tice, Ph.D.
Sponsor Study Monitor: LeRoy Metker

6.0 Primary Study Personnel:

Paul Andrews, M.S., Project Manager
Diane Satterfield, A.S., Research Assistant

7.0 Names and Addresses of Sponsor and Testing Facility:

Sponsor & Testing Facility: U.S. Army CHPPM
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Evaluation Facility - Integrated Laboratory Systems
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Durham, NC 27713

Mailing Address - Integrated Laboratory Systems
P.O. Box 13501
Research Triangle Park, NC 27709

8.0 Study Dates:

Study Initiation Date:	September 19, 1995
Evaluation Start Date:	November 20, 1995
Evaluation Termination Date:	January 9, 1996
Study Completion Date:	January 19, 1996

9.0 Experimental Design:

9.1 Treatment Schedule: The test article was administered at the U.S. Army exposure facility via inhalation for 6 hours/day (5 mice per sex in each of 7 test groups) on 3 consecutive days, followed by a single sample time 24 hours after administration of the final dose.

9.2 Dose Levels: 50% FE-13, 26% FE-13, 13% FE-13, 50% air/50% nitrogen, and 100% air.

9.2.1 Negative Controls: The negative controls consisted of a group of mice exposed to air only.

9.2.2 Positive Control: A separate group of animals was administered, by intraperitoneal injection, cyclophosphamide (25 mg/kg) dissolved in phosphate buffered saline.

9.3 Type of Evaluation:

9.3.1 Slide Preparation and Staining: Slide preparation and fixation was performed by U.S. Army personnel at the exposure facility. Once received at ILS, the smeared cells were stained with acridine orange (in sodium phosphate buffer, pH 7.4) for 5-10 minutes and then rinsed in buffer for 5-15 minutes. The slides were air dried and boxed in numerical order for scoring.

9.3.2 Scoring: Slides were scored at ILS in numerical order from randomly numbered animals so that the scorer did not have knowledge of the identity of the slide being analyzed. To assess if the test article inhibits the proliferation of the erythrocytes in bone marrow (signified by a reduction in the proportion of polychromatic erythrocytes within the total erythrocyte population), the number of PCEs among a total of 200 erythrocytes was determined per animal.

For micronuclei evaluation, 2000 PCEs/animal were evaluated in continuous field at 1000x magnification for the presence of micronuclei. Although a record was maintained of the number of micronuclei noted per PCE, the scored elements were the number of micronucleated cells, not the number of micronuclei.

9.4 Statistical Analysis: A two-way ANOVA was used to determine if a sex-dependent difference in response occurred at an alpha level of 0.05. Depending on the response obtained, male and female data were analyzed separately or pooled together. A one-tailed trend test based on the proportion of micronucleated cells among mice was used to determine if a treatment-related increase in DNA damage occurred at an alpha level of 0.05 (1,2). An ANOVA using individual animal responses was used to evaluate the effect of treatment on erythropoiesis. In addition, pairwise comparisons between each exposure group; and the corresponding control group was conducted using the appropriate statistical test (Pearson Chi-square test for micronuclei data or student's t test for percentage of PCE data). The statistical analysis of the micronuclei data was conducted using the Micronucleus Assay Data Management and Statistical Analysis software (1) developed by ILS for the EPA. In this program, a one-tailed trend test uses pooled data and incorporates a variance inflation factor to account for excess inter-animal variability.

10.0 Criteria for Determination of a Valid Test:

10.1 Dose Groups: The study must contain, per sex, a minimum of three groups (i.e.,

solvent control and two dose groups) with a minimum of three scorable mice per dose group.

10.2 Negative Control: The mean number of micronucleated PCE in the negative control group must not exceed 4%.

10.3 Positive Control: The positive control must induce a significant increase in the number of micronucleated PCE relative to the negative control.

10.4 Test Article: The test article, at least at the highest dose, should induce mortality (not to exceed 40%), animal toxicity, or bone marrow cytotoxicity (i.e., a significant depression in the percentage of PCE). However, if no mortality, animal toxicity, or cytotoxicity is observed at the limit of exposure of the test article, the assay will be considered acceptable.

11.0 Criteria for a Positive Response

The response to the test article will be deemed positive if the following criteria are met:

A significant, dose-dependent increase in the frequency of micronucleated PCE among mice is detected. This is demonstrated by a statistically significant ($P \leq 0.05$) finding in a one-tailed trend test.

A statistically significant ($P \leq 0.05$) increase in the frequency of micronucleated PCE is detected in at least one treatment dose, as indicated by a one-tailed student's t test comparison of each test dose against the concurrent solvent control treatment group.

If either, but not both, of the above conditions are met, the assay results will be classified as equivocal. If neither of the above conditions are met, the test article will be classified as negative for clastogenic activity in this *in vivo* test.

12.0 Records to be Maintained:

Scoring data were recorded on loose work sheets adapted or prepared as necessary for the test results. All data, slides, original copies of final report, and all correspondence will be archived at ILS until acceptance of the final report by the sponsor or three months after submission of the final report to the sponsor. At this time all items will be transferred to the sponsor for archiving.

13.0 Good Laboratory Practices Compliance:

The evaluation of the slides was conducted in accordance with Good Laboratory Practice regulations as promulgated by the Environmental Protection Agency (40 CFR Part 792).

14.0 Quality Assurance:

The protocol was reviewed by the ILS QAU before final approval. A quality assurance inspection of critical phases was conducted to assure the quality and integrity of the study results. An audit of the final report was conducted to determine the consistency between the reported information and the raw data.

15.0 Results:

15.1 Micronucleus Experiment: The MN experiment was conducted using doses of 13%, 26%, 50% FE-13, 50% air/50% nitrogen, and 100% air. Due to a limited number of inhalation exposure chambers, the study was conducted in two experiments. In the first experiment, mice were treated with 50% FE-13, 50% air/50% nitrogen, and 100% air. The purpose of the 50% air/50% nitrogen was to evaluate the effect of a decrease in oxygen on the endpoints being evaluated. In the second experiment, mice were treated with 26% FE-13, 13% FE-13, and 100% air. Cyclophosphamide was included as a positive control in both experiments. One of five female mice died in the positive control group in the second experiment. Bone marrow smears were prepared from the surviving mice 24 hours after the last treatment. Due to a difference between experiments in the mean MN-PCE frequencies of the 100% air control groups, control data were not pooled across experiments. Tables 1 through 4 present the individual mouse data per experiment, while Tables 5 through 8 provide group summary data per experiment.

Treatment with FE-13 did not result in a significant increase in the frequency of micronucleated PCE in either males ($P = 0.898$) or females ($P = 0.754$) for experiment 1 or males ($P = 0.426$) and females ($P = 0.175$) for experiment 2. Based on an ANOVA analysis, the percentage of PCE was not significantly depressed in males ($P = 0.602$) and females ($P > 0.800$) in experiment 1 or in males ($P = 0.740$) and females ($P > 0.800$) in experiment 2. In experiment 1, exposure to 50% air/50% nitrogen did not increase the frequency of micronucleated PCE in males ($P = 0.500$) or females ($P = 0.398$) and did alter the

percentage of PCE in males ($P = 0.024$) but not females ($P > 0.800$). The positive control, CP at 25 mg/kg, induced a significant increase in MN frequency in both experiments ($P < 0.001$) for both males and females with a significant depression in the percentage of PCE in males ($P = 0.007$) but not females ($P = 0.146$) in experiment 1 and in males ($P = 0.020$) but not females ($P = 0.064$) in experiment 2.

16.0 Conclusion:

Repeated inhalation with the test article FE-13 (trifluoromethane) did not significantly increase the frequency of micronucleated PCEs in the bone marrow of male or female B6C3F1 mice and or significantly affect the percentage of PCEs in either sex.

17.0 References:

1. Heddle, J.A., Hite, M., Kirkhart, B., Mavrournin, K., MacGregor, J.T., Newell, G.W. and Salamone, M.F. (1983) The induction of micronuclei as a measure of genotoxicity. A report of the U.S. EPA Gene-Tox Program. Mutat. Res. 123: 61-118.

2. Tice, R.R. and Ivett, J.L. (1985) Cytogenetic analysis of bone marrow damage. In: R.D. Irons (ed.) Toxicology of the Blood and Bone Marrow, Raven Press, NY, pp. 119-140.

Table 1: Individual Micronucleated PCE and %PCE Data for Male Mice Treated with FE-13 (Experiment 1)

DOSE (%)	ANIMAL NUMBER	MN-PCE/ 2000 PCE	%PCE
CP-25	255	52	56.0
mg/kg	258	43	49.0
	259	50	54.0
	262	62	62.5
	263	48	58.0
50%air/	254	12	74.5
50% N_2	267	4	76.5
	268	6	71.0
	269	6	72.0
	270	8	71.5
0	257	9	68.5
	260	7	73.5
	265	10	61.5
	271	5	66.0
	272	5	64.5
50	253	5	69.5
	256	6	69.5
	261	5	63.0
	264	7	77.5
	266	3	63.5

Table 2: Individual Micronucleated PCE and %PCE Data for Female Mice Treated with FE-13 (Experiment 1)

DOSE (%)	ANIMAL NUMBER	MN-PCE/ 2000 PCE	%PCE
CP-25	275	36	65.5
mg/kg	278	38	63.5
	282	41	68.5
	290	47	61.0
	292	41	70.0
50% air/	276	6	70.5
50% N_2	279	6	64.0
	284	11	74.0
	285	4	78.0
	288	4	61.5
0	273	3	65.5
	277	7	69.5
	280	6	69.0
	283	6	72.5
	289	7	68.0
50	274	4	76.5
	281	2	66.5
	286	3	65.0
	287	7	76.0
	291	8	64.0

Table 3: Individual Micronucleated PCE and %PCE Data for Male Mice Treated with FE-13 (Experiment 2)

DOSE (%)	ANIMAL NUMBER	MN-PCE/ 2000 PCE	%PCE
CP-25 mg/kg	315	43	62.0
	322	47	50.5
	326	41	62.0
	327	46	55.0
	331	52	42.0
0	313	1	75.0
	314	3	62.0
	316	6	61.0
	318	4	69.0
	328	2	74.0
13	319	0	61.0
	321	1	71.0
	323	3	73.0
	325	2	59.5
	332	4	73.5
26	317	4	64.0
	320	7	67.0
	324	1	71.5
	329	3	67.5
	330	2	56.0

Table 4: Individual Micronucleated PCE and %PCE Data for Female Mice Treated with FE-13 (Experiment 2)

DOSE (%)	ANIMAL NUMBER	MN-PCE/ 2000 PCE	%PCE
CP-25 mg/kg	293	49	44.5
	298 D		
	299	25	60.5
	307	28	64.5
	308	32	64.5
0	296	4	68.5
	297	3	69.5
	302	3	73.5
	305	1	78.5
	306	0	61.0
13	295	5	72.5
	303	4	64.5
	309	2	66.0
	311	2	75.5
	312	3	72.5
26	294	3	83.0
	300	6	73.0
	301	1	60.0
	304	4	71.5
	310	2	67.0

D = ANIMAL DIED

Table 5: Group Micronucleated PCE and %PCE Data for Male Mice Treated with FE-13 (Experiment 1)

DOSE (%)	MN-PCE/1000 PCE[a] MEAN	SEM	P-Value	%PCE[b] MEAN	SEM	P-Value	N
CP-25 mg/kg	25.4*	0.78	0.000	55.9*	1.11	0.007	5
50% air/50% N_2	3.6	0.34	0.500	73.1	1.04	0.024	5
0	3.6	0.26		66.8	2.02		5
50	2.6	0.17	0.898	68.6	2.63	0.602	5

[a] Group mean frequency of MN-PCE per 1000 PCE and standard error of the means among mice. Data based on 2000 PCE/mouse.
[b] Group mean percent PCE and standard error of the means among mice. Data based on 200 erythrocytes/mouse.
+ One-tailed trend or ANOVA test P-value for MN and %PCE data, respectively. Data analysis based on pooled cells.
* Significantly different at $p < 0.05$.

Table 6: Group Micronucleated PCE and %PCE Data for Female Mice Treated with FE-13 (Experiment 1)

DOSE (%)	MN-PCE/1000 PCE[a] MEAN	SEM	P-Value+	%PCE[b] MEAN	SEM	P-Value+	N
CP-25 mg/kg	20.3*	0.47	0.000	65.7	1.63	0.146	5
50% air/50% N_2	3.1	0.32	0.398	69.6	3.06	>0.800	5
0	2.9	0.18		68.9	1.13		5
50	2.4	0.29	0.754	69.6	2.74	>0.800	5

[a] Group mean frequency of MN-PCE per 1000 PCE and standard error of the means among mice. Data based on 2000 PCE/mouse.
[b] Group mean percent PCE and standard error of the means among mice. Data based on 200 erythrocytes/mouse.
+ One-tailed trend or ANOVA test P-value for MN and %PCE data, respectively. Data analysis based on pooled cells.
* Significantly different at $p < 0.05$.

Table 7: Group Micronucleated PCE and %PCE Data for Male Mice Treated with FE-13 (Experiment 2)

DOSE (%)	MN-PCE/1000 PCE[a] MEAN	SEM	%PCE[b] MEAN	SEM	N
CP-25 mg/kg	22.9*	0.47	54.3*	1.89	5
0	1.6	0.22	68.2	2.92	5
13	1.0	0.18	67.6	3.04	5
26	1.7	0.26	65.2	2.59	5
P-Value+	0.426		0.740		

[a] Group mean frequency of MN-PCE per 1000 PCE and standard error of the means among mice. Data based on 2000 PCE/mouse.
[b] Group mean percent PCE and standard error of the means among mice. Data based on 200 erythrocytes/mouse.
+ One-tailed trend or ANOVA test P-value for MN and %PCE data, respectively. Data analysis based on pooled cells.
* Significantly different at $p < 0.05$.

Table 8: Group Micronucleated PCE and %PCE Data for Female Mice Treated with FE-13 (Experiment 2)

DOSE (%)	MN-PCE/1000 PCE[a] MEAN	SEM	%PCE[b] MEAN	SEM	N
CP-25 mg/kg	16.8*	1.34	58.5	4.76	4
0	1.1	0.18	70.2	2.90	5
13	1.6	0.15	70.2	2.11	5
26	1.6	0.22	70.9	3.78	5
P-Value+	0.175		>0.800		

[a] Group mean frequency of MN-PCE per 1000 PCE and standard error of the means among mice. Data based on 2000 PCE/mouse.
[b] Group mean percent PCE and standard error of the means among mice. Data based on 200 erythrocytes/mouse.
+ One-tailed trend or ANOVA test P-value for MN and %PCE data, respectively. Data analysis based on pooled cells.
* Significantly different at $p < 0.05$.

APPENDIX

STUDY PROTOCOL & DEVIATIONS

STUDY PROTOCOL

1.0 Study Title:

Repeated Inhalation Exposure of FE-13 in Mice, *Mus musculus* (Bone Marrow Micronucleus Assay)

2.0 Study Identification:

ILS Project No. - A073-002
Sponsor Contract No. - DAAD05-91C-0018
U.S. Army Study No. - 85-3587

3.0 Purpose of the Study:

The objective of this study is to evaluate the polychromatic erythrocytes (PCE) of male and female B6C3F1 mice exposed via repeated inhalation to FE-13 (trifluoromethane) for the presence of micronuclei.

4.0 Names and Addresses of Sponsor and Testing Facility:

4.1	Sponsor:	U. S. Army ~~Environmental Hygiene Agency~~ Center for Health Promotion and Preventive Medicine Bldg. E-2100 Aberdeen Proving Ground, MD 21005
4.2	Testing Facility: (Exposure)	U.S. Army Center for Health Promotion and Preventive Medicine Bldg. E-2100 Aberdeen Proving Ground, MD 21005
	Testing Facility: (Evaluation)	Integrated Laboratory Systems
	Shipping Address:	800-12 Capitola Drive Durham, NC 27713
	Mailing Address:	P.O. Box 13501 Research Triangle Park, NC 27709

5.0 Proposed Study Dates:

Final Report Completion Date: November 10, 1995

6.0 Primary Study Personnel:

Leroy Metker, Sponsor Study Monitor

Raymond R. Tice, Ph.D., Study Director
Paul Andrews, M.S., Project Manager
Diane Satterfield, A.S., Research Assistant

7.0 Experimental Design:

7.1 Treatment Schedule: The test article will be administered at the U.S. Army exposure facility via inhalation for 6 hours/day (five mice per sex in each of 7 test groups) on three consecutive days, followed by a single sample time 24 hours after administration of the final dose.

7.2 Dose Levels: 50% air/50% FE-13, 50% air/50% nitrogen, 0.5x maximum concentration, 0.25x maximum concentration, and 1 additional exposure (if needed) to establish the maximum dose.

7.2.1 Negative Controls: The negative controls will consist of a group of mice exposed to air only.

7.2.2 Positive Control: A separate group of animals will be administered, by intraperitoneal injection, cyclophosphamide (25 mg/kg) dissolved in phosphate buffered saline.

7.3 Type of Evaluation:

7.3.1 Slide Preparation and Staining: Slide preparation and fixation will be performed by U.S. Army personnel at the exposure facility. Once received at ILS, the smeared cells are stained with acridine orange (in sodium phosphate buffer, pH 7.4) for 5-10 minutes and then rinsed in buffer for 5-15 minutes. The slides are air dried and boxed in numerical order for scoring.

7.3.2 Scoring: Slides will be scored at ILS in numerical order from randomly numbered animals so that the scorer does not have knowledge of the identity of the slide being analyzed. To assess if the test article inhibits the proliferation of the erythrocytes in bone marrow (signified by a reduction in the proportion of polychromatic erythrocytes within the total erythrocyte population), the number of PCEs among a total of 200 erythrocytes is determined per animal.

For micronuclei evaluation, 2000 PCEs/animal are evaluated in continuous field at 1000x magnification for the presence of micronuclei. Although a record is maintained of the number of micronuclei noted per PCE, the scored elements are the number of micronucleated cells, not the number of micronuclei.

7.4 Statistical Analysis: A two-way ANOVA is used to determine if a sex-dependent difference in response occurred at an alpha level of 0.05. Depending on the response obtained, male and female data are analyzed separately or pooled together. A one-tailed trend test based on the proportion of micronucleated cells among mice is used to determine if a treatment-related increase in DNA damage occurred at an alpha level of 0.05 (1,2). An ANOVA using individual animal responses is used to evaluate the effect of treatment on erythropoiesis. In addition, pairwise comparisons between each exposure group; and the corresponding control group will be conducted using the appropriate statistical test (Pearson Chi-square test for micronuclei data or student's t test for percentage of PCE data). The statistical analysis of the micronuclei data will be conducted using the Micronucleus Assay Data Management and Statistical Analysis software (1) developed by ILS for the EPA. In this program, a one-tailed trend test uses pooled data and incorporates a variance inflation factor to account for excess inter-animal variability.

8.0 Criteria for Determination of a Valid Test

8.1 Negative Control: The mean number of micronucleated PCE's in the control animals must not exceed 4%.

8.2 Positive Control: The frequency of micronucleated PCE's in the positive control group must be statistically increased relative to the negative control.

8.3 Test Article: A minimum of three groups (i.e., control and two dose groups) is required, and a minimum of three animals in any dose group must have survived and been evaluated. The test article, at least at the highest dose, should induce a significant depression in the mitotic index. However, if no cytotoxicity is observed at either the limit of exposure of the test article, the assay will be considered acceptable.

9.0 Criteria for Determination of Test Response

The response to the test article will be deemed positive if the following criteria are met:

A significant, dose-dependent increase in the percentage of micronucleated PCE's is observed, and

A statistically significant ($p \leq 5\%$) increase in the frequency of micronucleated PCE's in at least one dose group, as indicated by a one-tailed student's t-test comparison of the test group against the concurrent control.

If either, but not both, of the above conditions are met, the assay results will be evaluated by the Study Director and be classified as positive, negative, or equivocal depending on the nature and magnitude of the response.

If neither of the above conditions are met, the test article will be classified as negative for the micronucleus activity.

10.0 Records to be Maintained:

Scoring data will be recorded on loose work sheets adapted or prepared as necessary for the test results. All data, slides, original copies of the protocol and reports, and all correspondence will be archived at ILS until acceptance of the final report by the sponsor or three months after submission of the final report to the sponsor. At this time all items will be transferred to the sponsor for archiving.

11.0 Report:

A report of the results of this study will be prepared and will completely and accurately describe all methods used for the generation and analysis of the data.

12.0 Good Laboratory Practices Compliance:

The evaluation of slides will be conducted in accordance with Good Laboratory Practice regulations as promulgated by the Environmental Protection Agency in 40 CFR Part 792. The protocol will be reviewed by the ILS Quality Assurance Unit before final approval.

13.0 Quality Assurance:

At least one critical phase will be inspected during the evaluation process. An audit of the final report will be conducted to verify that reported values are supported by the raw data.

14.0 References:

1. Micronucleus Assay Data Management and Analysis System, Version 1.4 (1990) U.S. Environmental Protection Agency, Environmental Monitoring Systems Laboratory, Las Vegas, NV.

2. Margolin, B.H. and Risko, K.J. (1988) The statistical analysis of in vivo genotoxicity data. Case studies of the rat hepatocyte UDS and mouse bone marrow micronucleus assays, in "Evaluation of Short Term Tests for Carcinogens", Oxford University Press, Oxford, UK, pp 29-43.

15.0 Approvals

Sponsor (Study Monitor): LeRoy W Mitchell Date: 25 Aug 95

Study Director: Raymond Tice Date: Sept 19, 1995

INTEGRATED LABORATORY SYSTEMS

PROTOCOL DEVIATION

SPONSOR CODE: A1 PROTOCOL DEVIATION #: 1

ILS PROJECT NO.: A073-002 CHEMICAL REPOSITORY #: N/A

STUDY TITLE: Repeated Inhalation Exposure of FE-13 in Mice, *Mus musculus* (Bone Marrow Micronucleus Assay)

Deviation:

Under Protocol Section 8.3 - Criteria for a Valid Test, the second sentance should read: The test article, at least at the highest dose, should induce a significant depression in the percentage of PCE.

Cause of the Deviation:

The protocol mistakenly called for a depression in the mitotic index.

Corrective Action Taken:

None

Impact on Study:

None

Submitted by:

Raymond Tice 1-19-96

Study Director Date

USACHPPM

U.S. Army Center for Health Promotion and Preventive Medicine

Mutagenicity Testing of FE-13

Readiness Thru Health

Executive Summary

Since the fire extinguishant, Halon 1301 poses an atmospheric ozone depletion potential and environmental regulations no longer allow its production, a suitable replacement must be found. One product currently under examination as a replacement for Halon 1301 is FE-13. (Freon 23; trifluromethane; CHF_3) is a halogenated hydrocarbon considered to be chemically inert although it can release fluoride when exposed to flame or red-hot metal. The median lethal concentration (LC_{50}) of FE-13, based on a 4-hour exposure, is >650,000 ppm. A Toxicity Profile developed for The Army Program Executive Office, Armored Systems Modernization by the Toxicology Division, AEHA in 1994 indicated that no effects were observed for FE-13 in 90-day exposure regimes at 10,000 ppm (1%). The effective extinguishant concentration of FE-13 is 12%. The no observable adverse effects level (NOAEL), based on cardiac sensitization, for FE-13 is greater than 30%. The Toxicity Profile indicated that further testing was necessary to determine developmental, reproductive and mutagenicity potential.

Genotoxic testing is an important component of a toxicological profile. Compounds which induce alterations in nucleic acids and associated components are considered to be genotoxic. Mutagenic testing is a specific type of genotoxic testing. Mutagens can induce types of stable changes in the nucleotide sequence of genes, the chromosome structure, or the chromosome number. These types of genetic events are responsible for a large proportion of human genetic diseases and congenital defects.

The compound FE-13 was tested for its mutagenic potential using four separate test systems. Each test system examined a specific mutagenic component. These test procedures included both *in vivo* and *in vitro* assays.

The AS52/GPT mammalian mutagenesis assay examines a compound's ability to induce gene mutations in the genes which code for the enzyme guanine phosphoribosyl-transferase (*gpt*) of cultured AS52 Chinese hamster ovary cells[1]. The addition of the metabolic activator, S9, allows the identification of promutagens. This test procedure is capable of identifying agents which cause small and large deletion mutations as well as point mutations. Also, this assay can demonstrate the cytotoxicity of the compound by comparing the cloning efficiency of treated cultures with that of nontreated cultures. Cultures were exposed to air concentrations of FE-13 for five hours at 37°C. Concentrations of FE-13, with and without the S9 activator, were 10, 25, 50, 75, and 100%. Some cultures were exposed to 100% nitrogen in order to determine the effects of oxygen deprivation. FE-13 did not induce a significant level of mutagenicity in the presence or absence of metabolic activation.

The *Salmonella typhimurium* / microsome reverse mutation assay (Ames test), developed by Bruce Ames, is an elegant assay for the determination of mutagenicity[2]. This assay employs bacterial strains that are unable to manufacture histadine and is capable of detecting both base pair substitutions and frameshift mutations. The metabolic activator, S9, is used in this test to identify promutagens. The concentrations of FE-13 used in this test procedure were 10, 50, and 100% per plate. FE-13 did not induce a significant level of mutagenicity in the presence or absence of metabolic activation.

In vitro Chromosome aberrations can also be examined using Chinese hamster ovary (CHO) cells[1]. This assay is sensitive to clastogenic activity of a variety of chemicals. The

detection of a significantly elevated level of chromosome damage is considered an indicator of genetic damage. The S9 fraction of rat liver homogenate is also used in this test system to identify promutagens. Toxicity of FE-13 was examined in cultures, with and without S9, using concentrations of 5, 10, 25, 50, 75, and 100% and a four hour exposure period. One group was exposed to 100% nitrogen in order to determine the effects of oxygen deprivation. This procedure examined average generation time, mitotic index, polyploid index, and cell density. Clastogenic activity was evaluated using concentrations of 50, 60, 70, 80, 90, and 100% in the presence or absence of the S9 activator. As with the toxicity portion of this study, one group was exposed to 100% nitrogen in order to determine the effects of oxygen deprivation. A continuous exposure protocol in the absence of S9 was not practical due to the potential adverse effects of oxygen deprivation. FE-13 was found to induce a significant level of clastogenic damage in concentrations of 80% and above in the absence of metabolic activation. Control (air only) cultures containing S9 displayed a 2% (not statistically significant) increase in cellular damage while nonactivated cultures displayed no damage. This difference in baseline activity may have accounted for the nonsignificant increase in cellular damage with the S9 activator although the level of damage from exposure was identical with and without the S9 activator. Cells exposed to 100% nitrogen also displayed the same level of damage. Damage, therefore, is probably due to a decreased oxygen level rather than the activity of FE-13.

The mouse bone micronucleus assay is an *in vivo* test system which can determine the ability of a compound to induce micronuclei formation in immature erythrocytes of male and female mice[3]. Micronuclei are formed when chromosomes lag or fragment during cell division. The B6C3F1 strain of mouse was used in this study as this strain appears to be exquisitely sensitive to micronucleus induction. This assay is the most reliable method for evaluating the potential of a compound to induce clastogenic or aneugenic damage. FE-13 was assayed using concentrations of 13%, 26%, 50%. Control animals were exposed to 100% air as well as an oxygen poor environment of 50% air and 50% nitrogen. FE-13 did not induce a significant level of mutagenicity.

The results of the above tests indicate that FE-13 does not induce a mutagenic effect at dosage levels tested and, from a mutagenicity standpoint, it appears to be a suitable replacement for Halon 1301. Further genotoxicity testing of this material is not indicated at this time.

References

1. Preston, R.J., W. Au, M.A. Bender, J.G. Brewen, A.V. Carrano, J.A. Heddle, A.F. McFee, S. Wolf, and J.S. Wassom. (1981). Mammalian *in vivo* and *in vitro* cytogenic assays: A report of the U.S.E.P.A. Gen-Tox Program. Mutation Res. 87:143-188.

2. Ames, B.N., J. McCann, and E. Yamasaki. (1975). Methods for detecting carcinogens and mutagens with the Salmonella mammalian microsome mutagenicity chromosome. Mutation Res. 31:347-364.

3. Heddle, J.A., M. Hite, B. Kirkhart, K. Mavrournin, J.T. MacGregor, G.W. Newell, and M.F. Salamone. (1983). The induction of micronuclei as a measure of genotoxicity. A report of the U.S.E.P.A. Gene-Tox Program. Mutation Res. 123:61-118.

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